Adeno-associated virus type 2 Rep68 can bind to consensus rep-binding sites on the herpes simplex virus 1 genome

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Abstract: Adeno-associated virus type 2 is known to inhibit replication of herpes simplex virus 1 (HSV-1). This activity has been linked to the helicase- and DNA-binding domains of the Rep68/Rep78 proteins. Here, we show that Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome and that the Rep helicase activity can inhibit replication of any DNA if binding is facilitated. Therefore, we hypothesize that inhibition of HSV-1 replication involves direct binding of Rep68/Rep78 to the HSV-1 genome.

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Short-Form Paper

AAV2 Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome

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ABSTRACT

Adeno-associated virus type-2 is known to inhibit replication of herpes simplex virus type-1 (HSV-1). This activity has been linked to the helicase- and DNA-binding domains of the Rep68/78 proteins. Here, we show that Rep68 can bind to consensus Rep-binding sites on the HSV-1 genome and that the Rep-helicase activity can inhibit replication of any DNA if binding is facilitated. Therefore we hypothesize that inhibition of HSV-1 replication involves direct binding of Rep68/78 to the HSV-1 genome.

Keywords: AAV2; HSV-1; Rep-binding site; Rep helicase.

FINDINGS

Adeno-associated virus type-2 (AAV2) is a nonpathogenic human parvovirus with a unique biphasic life cycle. In the absence of a helper virus, AAV2 establishes a latent infection while in the presence of a helper virus, such as adenovirus type-2 (AdV2), herpes simplex virus type-1 (HSV-1) or human papillomavirus type-16 (HPV-16), it undergoes lytic replication (1–4). The AAV2 genome is a single-stranded (ss) DNA of 4,680 nucleotides, which is packaged into an icosahedral capsid with a diameter of approximately 20 nm (5). The genome harbors two clusters of genes, *rep* and *cap*, which are flanked by inverted terminal repeats (ITRs). The ITRs form hairpin structures and contain a Rep-binding site (RBS) and a terminal resolution site (trs), which together act as viral origin of DNA replication (6, 7). The *cap* gene is transcribed from the p40 promoter and encodes the capsid proteins VP1, VP2, and VP3, which differ in their N-termini due to alternative start codons (8, 9). In addition, a nested open-reading frame (ORF) within the *cap* gene encodes a protein designated assembly-activating protein (AAP), which is believed to be required for AAV2 capsid assembly in the nucleolus (10, 11). The *rep* gene encodes the Rep proteins, which are synthesized in four different forms due to transcription from two different promoters, p5 and p19, and alternative splicing of an intron near the C-terminal end (12). The different Rep proteins are termed Rep40, Rep52, Rep68, and Rep78 according to their apparent molecular weight. The Rep proteins are involved in diverse processes during the viral life cycle, such as DNA replication, regulation of gene expression, genome packaging, and site-specific genomic integration (13–18).

HSV-1 belongs to the subfamily of the *Alphaherpesvirinae* and is the reagent causing mucosal eruptions at the site of infections, which can reoccur at the same site upon reactivation from latency (19, 20). The HSV-1 virion is built up by three structural components which are the capsid, the tegument and the surrounding envelope. The viral genome is a linear double stranded DNA molecule of 152 kb in size and has a unique structure. It is divided into two covalently joined segments, which contains unique segments (U_L, U_S) and inverted repeat regions (TR_L, IR_L, IR_S, and TR_S). The IR sequences link the L

and S segments (Fig. 1A). HSV-1 gene expression and replication occurs in a temporally regulated cascade; immediate early (IE), early (E) and late (L). IE proteins mainly exhibit regulatory functions and initiate expression of the E genes. The E proteins comprise enzymes necessary for viral DNA replication and are therefore required for the expression of some of the L genes, as expression of these genes relies on DNA replication. All viral replication events take place in the nucleus within distinct areas termed replication compartments (RCs) (21). In the course of viral DNA replication, these RCs grow continuously and four different stages (I-IV) can be distinguished according to RCs staining patterns (22–24). The minimal set of HSV-1 proteins required for initiating AAV2 replication consists of the E proteins UL5, UL8, and UL52, which together form the HSV-1 helicase/primase complex, as well as the ssDNA binding protein ICP8 (UL29) (25–27). In addition, the HSV-1 IE proteins ICP4 and ICP0, the E protein complex forming the HSV-1 polymerase (UL30 and UL42) and the US1 gene product, strongly enhance AAV2 replication (26). AAV2 has developed strategies to inhibit helper virus replication, likely to reduce competition (24, 28–33). For example, expression of the AAV2 non-structural proteins Rep68/78 alone leads to a significant inhibition of HSV-1 DNA replication (24, 28). Specifically, we demonstrated that the AAV2 Rep protein domains responsible for the inhibition of HSV-1 DNA replication include the DNA-binding and the ATPase/helicase activities, while the endonuclease activity is not required (28). We also showed that Rep-mediated inhibition of HSV-1 occurs even in absence of AAV DNA and is not due to alterations of HSV-1 immediate-early (IE) and early (E) gene expression, nor due to the Rep-mediated induction of toxic stress in the cell, but rather occurs at the stage of HSV-1 DNA replication itself (28). We hypothesized that a possible mechanism of Rep68/78-mediated inhibition of HSV-1 DNA replication may involve binding of Rep proteins to consensus Rep-binding sites (RBS) on the HSV-1 genome and modification of the bound DNA substrate via the Rep helicase activity. To investigate this possibility, we now addressed the following two questions: (i) do consensus RBS exist on the HSV-1 genome and if so, can AAV2 Rep

proteins bind to these sites and (ii) can the AAV2 Rep helicase activity inhibit replication of any DNA substrate when binding is facilitated?

We addressed the first question by screening the wild type (wt) HSV-1 (strain F) genome for the minimal consensus RBS motif GAGYGAGC as a prerequisite for the ability of Rep to specifically bind to dsDNA templates via its DNA-binding domain (34). We found that such sites are indeed present as shown in Fig. 1. All consensus RBS are located within coding sequences of genes found in the unique long (U_L) segment of the HSV-1 genome (Fig. 1A). Sequence alignment revealed that all putative HSV-1 RBS (pRBS) consist of two complete GAGC repeats with the exception of pRBS No. 7, which contains a T in place of a C at position 74604 (nt) but still complies with the GAGYGAGC consensus sequence (Fig. 1B). Importantly, no consensus trs (CCAACT) (6, 18) was found within 8-13 nt after any putative HSV-1 RBS, excluding the existence of a AAV2 integration site at these positions on the HSV-1 genome (35). We next tested the capability of AAV2 Rep proteins to bind via the DNA-binding domain to putative HSV-1 RBS by electrophoretic mobility-shift assays (EMSA) using purified His-tagged Rep68 proteins (His-Rep68). We designed 37-mer duplexed oligonucleotides harboring selected putative RBSs (No. 1-5; Fig. 1B, light grey box), which were radioactively labelled with [γ-^{32}P]-ATP. An oligonucleotide containing the native RBS from the AAV2 inverted terminal repeat (ITR) was used as the positive control. An oligonucleotide containing a random sequence from within the UL44 gene harboring no putative RBS was used as negative control. Approximately 5 fmol of each duplexed oligonucleotide was incubated with 0, 120, or 240 ng of His-Rep68 protein for 30' at room temperature (RT) and then subjected to 4% polyacrylamide gel electrophoresis. After 3h, the gel was dried and exposed to Fujifilm Imaging Plates, which were developed with a Fujifilm FLA-7000 Image Plate reader. For all oligonucleotides No. 1-5 examined we observed a dose-dependent shift compared to the unbound DNA template (Fig. 2A). The %-shift values for the oligonucleotides No. 1-5 were slightly lower (63-76%) than that of the positive control (ITR) (87%). The negative control oligonucleotide (nc) was shifted 5% only. Multiple shifted bands of the oligonucleotides No. 1-5 as well as the ITR-oligonucleotide were observed,

possibly due to the different oligomerization states Rep68 can form on ds DNA templates (36–38). To confirm that binding of His-Rep68 to the pRBS is indeed specific, we performed EMSAs as described above, except that 5 fmol of non-labeled (cold) duplex ITR oligonucleotide was used as competitor. The cold ITR competitor DNA appeared to prevent binding of His-Rep68 to the pRBS oligonucleotides, as no shift was observed under these conditions for the oligonucleotides No. 1-3 and a clearly reduced shift for the oligonucleotides No. 4-5 (Fig. 2B).

In order to investigate whether AAV2 Rep68 is able to bind to consensus RBS on the HSV-1 genome also in HSV-1 infected cells, we performed chromatin-immunoprecipitation (ChIP) assays followed by quantitative (q)PCR analysis (ChIP-qPCR). For this, Vero cells were transfected with plasmids expressing either Rep68, or Rep52 proteins fused with enhanced green fluorescent protein (EGFP). The next day, the cells were infected with wt HSV-1 (strain F) at an MOI of 40. At 16 h after infection, the cells were fixed with 4% paraformaldehyde (PFA), sonicated for 10min and processed for ChIP using the GFP-Trap® kit (Chromotek). The immunoprecipitated DNA oligonucleotides were then analyzed by qPCR using primers specific for the consensus RBS No. 1-9 (Fig. 1 and Table. 1). Primers for amplification of a sequence from the HSV-1 genome containing no proximal RBS (US1) served as a negative control (Table. 1). The qPCR mix was the following: 0.25µl of each primer [10µM], 10µl of SYBR® Green mix (SYBR® Green PCR Master Mix, Applied Biosystems) and 2.5µl of DNA in a final volume of 20µl. The reaction was carried out as follows: 95°C for 3min, 39x (95°C for 15sec, 60°C for 1min) followed by a final elongation step at 95°C for 10min. The raw Ct values were analyzed using the percent input (%-INPUT) method ($\%\text{-INPUT} = 100*2^{\Delta Ct}$, whereas ΔCt = (Ct [Input] - Log2 (Input Dilution Factor) - Ct [ChIP]). For each primer pair tested (no RBS and putative RBS 1-9), the %-INPUT values were calculated from cells expressing either Rep52-GFP (light grey bars) or Rep68-GFP (dark grey bars) (Fig. 2C). Using this data we next addressed (i) whether Rep68 binding to the HSV-1 DNA is more efficient at a pRBS and (ii) whether the DNA-binding domain of Rep68 is required for binding.

To address the first point, we calculated the difference between each value obtained from binding of Rep68-GFP to a pRBS and the control value (no RBS) which was set as 1. The graph in Fig. 2D shows that Rep68-GFP can bind more efficiently to the HSV-1 DNA that harbours a pRBS than to the control with no pRBS, with one exception; binding to pRBS No. 7 was not more efficient than binding to the control with no pRBS, likely because the pRBS No. 7 consensus sequence is slightly aberrant (Fig. 1B). Nevertheless, we can conclude that binding of Rep68 to HSV-1 DNA is more efficient when it harbors a pRBS. To address the second point, we calculated the difference (%-INPUT ratio) between the Rep68-GFP and the Rep52-GFP values for each consensus binding site No. 1-9 and the negative control (no RBS). The ratios were normalized to the no RBS (US1) control ratio and are shown in Fig. 2D. As expected, the ratio between Rep68-GFP and Rep52-GFP was the smallest in the absence of a consensus RBS (no RBS) and was set as 1. The ratios for all pRBS No. 1-9 were clearly higher and were statistically significant for pRBS No. 3-8. We can therefore conclude that the DNA-binding domain of Rep68 enhances binding to pRBS in this assay. To appreciate the quality of the binding of Rep68 to pRBS, we tested binding of a HSV-1 DNA binding protein, ICP4, to the ICP4-binding site in the HSV-1 ICP0 promoter relative to an unspecific DNA that does not contain an ICP4 binding site (US1). For this, we infected Vero cells with a recombinant HSV-1 (rHSVEYFP-ICP4) which expresses the ICP4 protein fused to the enhanced yellow fluorescent protein (EYFP) (39). One day later, we processed the cells for ChIP and qPCR as described above. We found that the ICP4 protein binds specifically to the ICP0 promoter. Binding efficiency was approximately 2.5-fold higher to the ICP0 promoter sequence than to the US1 control sequence (Fig. 2F) and is comparable to previous findings (40). We can therefore conclude that binding of AAV2 Rep68 to the HSV-1 DNA at a pRBS is at least as efficient as binding of the native HSV-1 DNA-binding protein ICP4 to its binding site in the HSV-1 ICP0 promoter (Fig. 2F).

To investigate the question whether the AAV2 Rep helicase domain can inhibit replication of any DNA substrate when binding is facilitated, we used a well-established assay to investigate the replication of HSV-1 and AAV2 DNA (21, 24, 41, 42). Briefly, cells are co-

transfected with two plasmids; the first contains 40 binding sites for the lac repressor protein LacI and an HSV-1 origin of DNA replication (pHSV-lacO), and the second plasmid encodes the *lac*-operon binding motif of LacI fused with enhanced yellow fluorescent protein (EYFP-LacI). In presence of HSV-1 helper functions, pHSV-lacO replicons (and therefore the binding sites for LacI) are amplified (because of the HSV-1 origin of DNA replication) and recruitment of autofluorescent LacI protein then allows visualization of pHSV-lacO DNA replication. We have now modified this assay by transfecting a third plasmid encoding the AAV2 Rep40/52 proteins, which contain the helicase but not the DNA binding domain, fused with the *lac*-operon binding motif of LacI (Rep40/52-LacI) (Fig. 3, A and B). The modified Rep40/52 proteins are prone to bind to *lac*-operon binding sequences present on the pHSV-lacO replicon and therefore allowed us to study the effect of the Rep helicase activity independent of the interaction between the AAV2 Rep DNA-binding domain and the RBS. The results shown in Fig. 3 (C-E) demonstrate that the Rep40/52-LacI proteins were indeed able to inhibit the replication of pHSV-lacO, as cells containing mature stage IV replication compartments (RCs) were not observed (Fig. 3C, panel n). By contrast, the Rep40/52 proteins, which lack any DNA binding domain, did not prevent the formation of mature stage IV HSV-1 RCs (Fig. 3C, panel i). Neither did Rep40/52K340H-LacI proteins, which can bind to the pHSV-lacO replicon but contain a point mutation that inactivates the helicase activity (43), although the numbers of stage IV RCs was reduced by approximately 50%, indicating that these fusion proteins can inhibit the replication of the HSV-1 replicon to some extent (Fig. 3C panel s). Also, in the cultures transfected with the Rep40/52-LacI encoding plasmid the frequency of stage III RCs was reduced by approximately 3-fold (Fig. 3C, panel m) while the frequency of cells showing diffuse EYFP-fluorescence (stage I; no RCs) was much higher when compared with the control cultures: no Rep (Fig. 3C, panel a), Rep40/52 (Fig. 3C, panel f) and Rep40/52K340H-LacI (Fig. 3C, panel p). We confirmed that Rep40/52-LacI proteins indeed bind to LacI-repressor binding sites on the pHSV-lacO reporter DNA by determining the degree of colocalization of the LacI-EYFP and the Rep AF594 signals in the merged images of panels l-n and q-s (Fig. 3E). The colocalization values in the smaller RCs

(stage II and III) were lower in trend then the values of the mature RCs (stage IV). This observation may be explained as follows: at early time points when the numbers of pHSV-lacO replicons are small, there is more competition for Lac-repressor binding sites between the Rep-LacI construct and the LacI-EYFP reporter protein. The distribution of stage I-IV RCs was comparable in cells transfected with the Rep40/52 (Fig. 3C, panels f-i) and the Rep40/52K340H-LacI (Fig. 3C, panels p-s) encoding plasmids, indicating that Rep40/52-LacI competition with EYFP-LacI did not interfere with detection of RCs. Moreover, while Rep68/78 can efficiently inhibit wtHSV-1 DNA replication (24, 28), Rep40/52, Rep40/52-LacI, and Rep40/52K340H-LacI do not (reference 28 and data not shown). These results indicate that the AAV2 Rep helicase activity in absence of the Rep DNA binding and endonuclease activities can inhibit the replication of any DNA template as long as it can bind to it. Of note, we have observed 5.3±9.2% and 17.3±4.6% of cells showing numerous nuclear foci when transfected with Rep40/52-LacI (Fig. 3C, panel o) or the helicase mutant Rep40/52K340H-LacI (Fig. 3C, panel t) respectively. Such foci were not observed when cells were transfected with the Rep40/52 (Fig. 3C, panel j) encoding plasmid or the empty backbone plasmid pcDNA3.1+ (Fig. 3C, panel e). Some of the foci represent background binding of EYFP-LacI to Lac-repressor binding sites on the pHSV-lacO input plasmid because foci were observed also in presence of pHSV-lacO and EYFP-LacI and absence of helpervirus (not shown). This hypothesis is supported also by the fact that Rep52-LacI proteins, which can also bind to the Lac-repressor binding sites on the pHSV-lacO replicons, co-localized with the numerous EYFP-LacI foci (Fig. 3C, insets panel o and t). While we do not know why the number of foci increases markedly in presence of Rep40/52-LacI constructs or absence of HSV-1 helpervirus, similar patterns have been observed before to occur when HSV-1 replication is inhibited by treating the cells with viral polymerase inhibitors such as phosphonoacetic acid (PAA), transfection of Rep68/78 encoding plasmids, or by use of polymerase-deficient HSV-1 (24, 44, 45).

In this report we demonstrate that the AAV2 Rep68 protein is capable of binding to pRBS on the ds HSV-1 genome *in silico*, *in vitro*, and in HSV-1 infected cells. EMSA and ChIP-qPCR

data revealed that binding of Rep68 to consensus RBS is specific and requires the Rep DNA-binding domain. Moreover, Rep68 is not able to bind efficiently to random HSV-1 sequences (UL44 and US1). In addition, we showed that the Rep helicase activity can inhibit replication of a random DNA substrate if binding is facilitated.

Generally, replicative stress is rapidly sensitized in the cell by numerous stress response factors and pathways such as DNA-damage response (DDR) or DNA-damage tolerance (DDT) (Reviewed in 46). One of the best understood stress responses resembles the DDR pathway initiated by the sensor-kinase ataxia-telangiectasia mutated (ATM)- and Rad3-related (ATR), which is activated upon stalling of the replication fork (RF). At a stalled RF, the helicase activity of the mini-chromosome maintenance protein complex (e.g. MCM2) is uncoupled from the replication complex and continues unwinding, which is generating a stretch of ssDNA recognized and covered by the replication protein A (RPA) (47–50). This leads to the activation of ATR, which then induces a DDR resulting in cell-cycle arrest allowing the cell to resolve the stalled RF (51–55). However, persisting replication stress can result in the collapse of the RF, associated with double-strand breaks (DSBs) and the consecutive inhibition of DNA replication (56).

Hence, we hypothesize that binding of Rep to consensus RBS and the helicase activity may generate a situation resembling a stalled RF, in particular when the Rep helicase activity is uncoupled from the replication activity, either because of the absence of a functional trs or the absence of the endonuclease activity. Support for this theory comes from previous observations which showed that the AAV2 Rep68/78 proteins induce a cellular DDR which is characterized by the activation of RPA and ATM, leading to an S-phase arrest (28, 57–59). It was initially hypothesized that the activation of ATM is a response to nicks induced by the endonuclease activity of Rep at multiple trs located on the cellular chromatin (59). However, we have shown in a later study that the activation of RPA and ATM also occurs with a Rep68/78 mutant lacking endonuclease activity (Y156F) and that the DNA-binding and ATPase/helicase activities of Rep are necessary for activation of these DNA damage

markers (28). We therefore hypothesize that the activation of RPA and ATM might in fact be due to Rep-induced dsDNA breaks caused by persistent stalling of RFs, although this possibility remains to be investigated.

It has been shown that the AAV2 Rep proteins are also capable of binding to the DNA of other helperviruses, such as AdV. In particular, Rep68 proteins can bind specifically to the AdV E2A promoter (60) as well as to a 55-bp DNA fragment within the AdV major late transcription promoter (MLP) via interaction with the cellular TATA box-binding protein (TBP) (61). These interactions of Rep68 with the AdV genome are mediating inhibition of transcription and therefore may directly affect AdV replication. However, in contrast to HSV-1, binding of Rep68 to the AdV genome occurs independently of consensus RBSs, and inhibition of AdV replication does not imply the Rep helicase activity. Whether the AAV2 Rep proteins are capable of binding to the HSV-1 genome independently of a consensus RBSs, e.g. via viral or cellular proteins that bind to the HSV-1 DNA, is a matter of current investigations. Of note, the Rep40/52 proteins, which have no DNA-binding domain, are not capable of binding to double-stranded (ds) DNA templates, neither to consensus RBS nor to random sequences. However, Rep40/52 can very well bind to single-stranded (ss) DNA templates, which is facilitated by two lysine residues (Lys-404 and Lys-406) located within the helicase domain (62). We indeed observed some unspecific binding of Rep52 to consensus RBS within the HSV-1 genome (Fig. 2C), which may occur when stretches of ssDNA are exposed naturally during replication of the HSV-1 genome. However, since Rep40/52 alone is not capable of inhibiting HSV-1 DNA replication (28), we consider that unspecific binding of Rep40/52 to consensus RBS on the HSV-1 genome may not exhibit sufficient binding capacity to inhibit HSV-1 DNA replication.

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FIGURE LEGENDS

Figure 1. The HSV-1 genome contains nine putative Rep-binding sites (pRBS). (A) The minimal AAV2 Rep-binding site motif GAGYGAGC was used to identify consensus Rep-binding sites within the HSV-1 (strain F) genome, that can be divided into unique long (U_L) and unique short (U_S) segments which are both flanked by internal and terminal inverted repeats (TR_L, IR_L, IR_S, TR_S). The arrowheads facing down represent pRBS located on the plus-strand and the arrowheads facing up represent pRBS located on the minus-strand. The pRBS were numbered randomly from 1 to 9 as indicated. The HSV-1 genes containing a consensus pRBS are highlighted. (B) Alignment of sequences from the AAV2 ITR and the nine HSV-1 pRBS identified in panel A. The consensus RBS (motif GAGYGAGC) and the trs located on the AAV2 ITR are indicated (dark grey boxes). Sequences analyzed in the in vitro experiments No. 1-5 are squared in a light grey box. The illustration was generated using the *prettyplot* function of the online tool EMBOSS (http://pro.genomics.purdue.edu/emboss/). The numbers on the right indicate the nucleotide (nt) position within the AAV2 genome (ITR) or the HSV-1 genome No. 1-9. Conserved nucleotides are indicated below as a schematic representation created with *weblogo* (63).

Figure 2. Rep-binding assays. (A) Electrophoretic mobility shift assays (EMSA) were performed with purified His-tagged Rep68 proteins (His-Rep68) and radiolabeled duplex oligonucleotides No. 1-5 containing pRBS, the negative control (nc) or the positive control (ITR); see light grey box in Fig. 1B for oligonucleotide sequences. The amount (ng) of His-Rep68 protein used is indicated at the top of the blot. (B) EMSA competition assays were performed with cold competitor DNA consisting of the positive control oligonucleotide (ITR), presented at equal-fold molar excess. The %-shift values were calculated for all the reactions and are shown below the blots. (C) Chromatin immuno-precipitation (ChIP) assays followed by qPCR (ChIP-qPCR) were performed with Vero cells (approx. $1.5x10^6$ cells in a 6 cm cell culture dish) transfected with 0.5µg of a plasmid encoding either the Rep68-EGFP fusion protein (Rep68-GFP) or the Rep52-EGFP fusion protein (Rep52-GFP) and superinfected

with wtHSV-1 (strain F) at an MOI of 40. At 16 h after infection the cells were processed for ChIP as described in the text. The precipitated DNA was analyzed by qPCR using specific primers flanking the pRBS No. 1-9 on the HSV-1 genome or primers flanking a sequence of the HSV-1 US1 gene that contains no proximal pRBS (no RBS); Table.1. The data was quantified using the percentage input (%-INPUT) method as described in the text. (D) The Rep68-GFP values from panel C were normalized to the *no RBS*-control, which was set as 1. (E) The ratio between the Rep68-GFP and the Rep52-GFP values was calculated and normalized to the ratio of the control values (no RBS). Bars represent mean values and standard errors (SE) from 3-6 individual experiments. (F) Vero cells were infected with rHSVEYFP-ICP4 at an MOI of 5. Two days later, the cells were processed for ChIP-qPCR as described in the text. The qPCR was performed with primers flanking the ICP0 promoter (ICP0 prom.) or the US1 locus as a negative control. The resulting Ct values were analyzed using the percent input (%-INPUT) method. The results are shown as %-INPUT values (left) and the normalized %-INPUT values (right). Asterisks indicate statistically significant differences between the negative controls (no RBS/US1) and the corresponding binding sites on a paired two-tail students t-test (* = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$).

Figure 3. Inhibition of DNA replication by the AAV2 Rep helicase activity. (A) Schematic representation of the Rep constructs analyzed in this experiment. The functional protein domains of interest for this study are indicated. The arrow facing down indicates the splicing site of the Rep gene. (B) Western analysis of Rep40/52, Rep40/52-LacI, and Rep40/52K340H-LacI. Vero cells (200'000 cells/well) were transfected with 0.1µg of a plasmid encoding either Rep40/52, Rep40/52-LacI, Rep40/52K340H-LacI, or the empty plasmid backbone pcDNA3.1+ (no Rep). After 24h the cells were harvested and processed for Western blotting using a Rep-specific antibody (mouse-anti Rep-mAb, clone 303.9, Fitzgerald; 1:100). Actin staining served as loading-control. (C) HSV-1 DNA replication assay. Vero cells (150'000 cells/well) were co-transfected with 0.05µg of the HSV replicon plasmid pHSV-lacO, 0.01µg of the reporter plasmid pSV2EYFP-LacI and 0.05µg of a plasmid encoding either Rep40/52-LacI (panel k-o), the helicase deficient mutant Rep40/52K340H-

LacI (panel, p-t), wtRep40/52 (panel f-j) or the empty plasmid backbone pcDNA3.1+ (no Rep) (panel a-e). One day after transfection the cells were superinfected with wt HSV-1 (strain F) at an MOI of 5. After 16 h, the cells were fixed with 4% paraformaldehyde (PFA), immuno-stained for Rep with a primary mouse (ms) anti Rep-mAb (clone 303.9, Fitzgerald, 1:100) and a secondary goat (gt) anti-ms IgG(H+L)–Alexa Fluor 594 (Molecular Probes®, 1:500) (red insets), and then subjected to confocal laser scanning microscopy with an SP2 CLSM from Leica. The percentage of cells displaying pHSV-lacO replication compartments at stage I, II, III or IV as well as the number of cells showing numerous foci are indicated. Scale bar = 5µm (D) Graph represents the data shown in panel C. Error bars show standard deviation (SD) from three independent experiments with 80-100 cells counted in each experiment. Asterisks indicate statistically significant differences based on a paired two-tail students t-test (* = $p<0.05$; ** = $p<0.01$). (E) The panels l-n and q-s from Fig. 3C are shown as merged images. The blue insets represent the DAPI stain of the corresponding cells. The degree of colocalization [%] was calculated using the *coloc*-function of the software Imaris® (Bitplane). Abbreviation: n/o = not observed.

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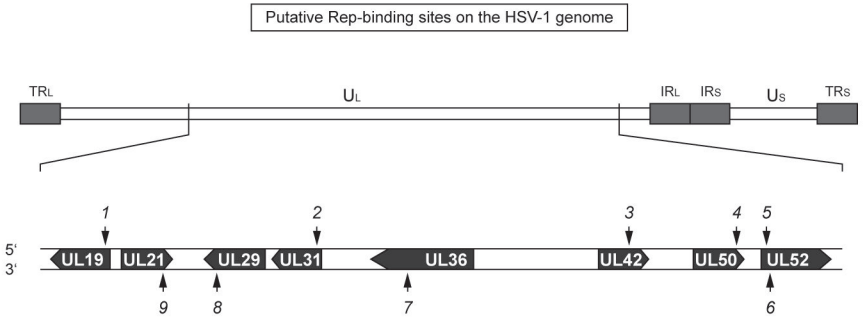
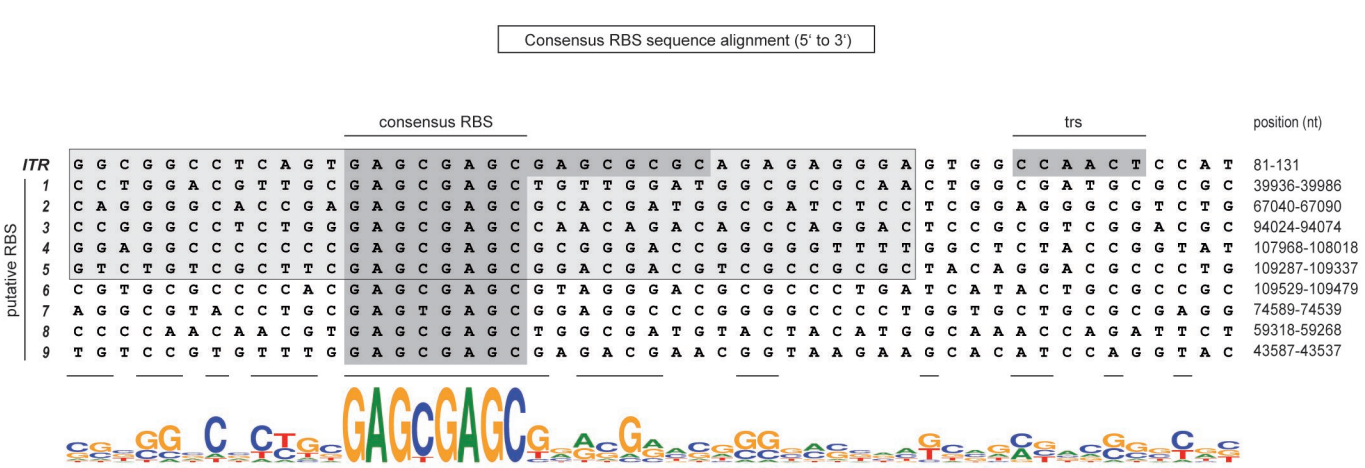
A


Putative Rep-binding sites on the HSV-1 genome
TRL
UL
IRL
IRS
US
TRS
1
2
3
4
5
5'
3'
UL19
UL21
UL29
UL31
UL36
UL42
UL50
UL52
3'
5'
9
8
7
6

B


Consensus RBS sequence alignment (5' to 3')
consensus RBS
trs
position (nt)
putative RBS
ITR G G C G G C C T C A G T G A G C G A G C G A G C G C G C A G A G A G G G A G T G G C C A A C T C C A T 81-131
1 C C T G G A C G T T G C G A G C G A G C T G T T G G A T G G C G C G C A A C T G G C G A T G C G C G C 39936-39986
2 C A G G G G C A C C G A G A G C G A G C G C A C G A T G G C G A T C T C C T C G G A G G G C G T C T G 67040-67090
3 C C G G G C C T C T G G G A G C G A G C C A A C A G A C A G C C A G G A C T C C G C G T C G G G A C G C 94024-94074
4 G G A G G C C C C C C C G A G C G A G C G C G G G A C C G G G G G G T T T T G G C T C T A C C G G T A T 107968-108018
5 G T C T G T C G C T T C G A G C G A G C G G A C G A C G T C G C C G C G C T A C A G G A C G C C C T G 109287-109337
6 C G T G C G C C C C A C G A G C G A G C G T A G G G A C G C G C C C T G A T C A T A C T G C G C C G C 109529-109479
7 A G G C G T A C C T G C G A G T G A G C G G A G G C C C G G G G C C C C T G G T G C T G C G C G A G G 74589-74539
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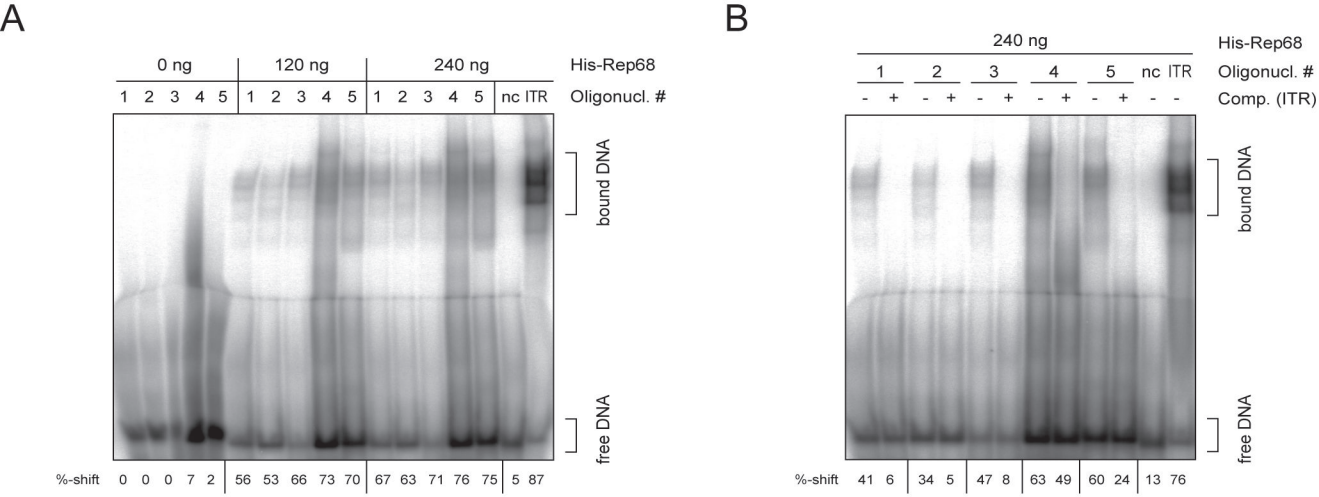
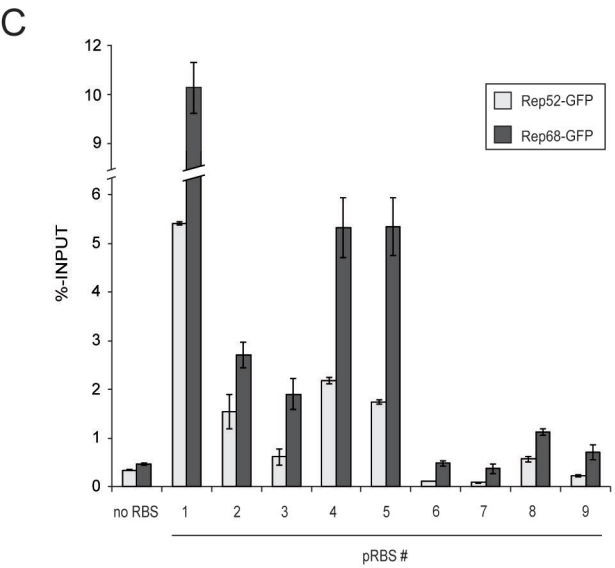
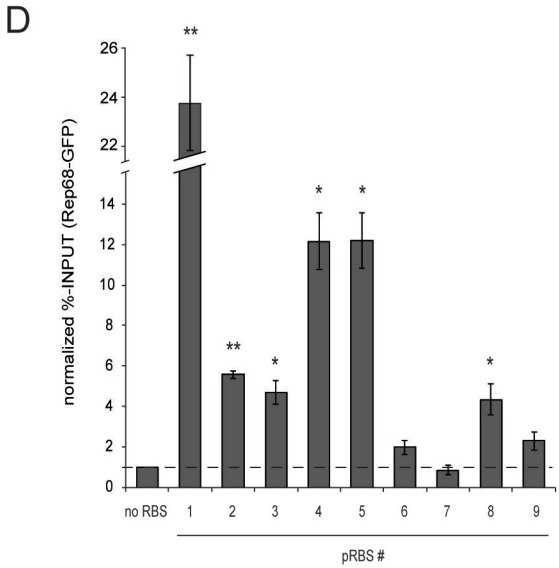
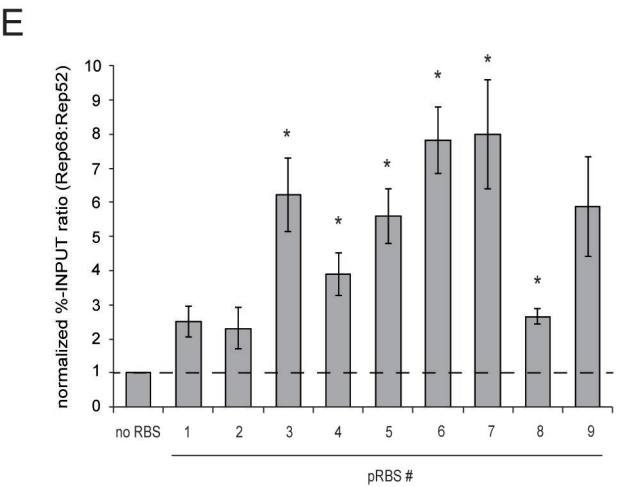
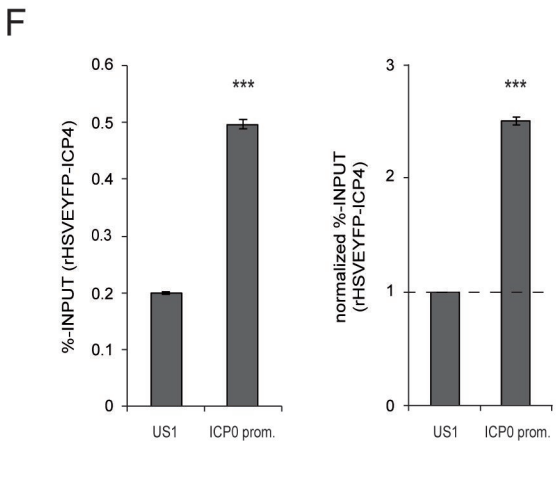

A
0 ng
120 ng
240 ng
His-Rep68
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5 nc ITR
Oligonucl. #
bound DNA
free DNA
%-shift 0 0 0 7 2 56 53 66 73 70 67 63 71 76 75 5 87

B
240 ng
His-Rep68
1 2 3 4 5 nc ITR
Oligonucl. #
- + - + - + - + - + - -
Comp. (ITR)
bound DNA
free DNA
%-shift 41 6 34 5 47 8 63 49 60 24 13 76


C
Rep52-GFP
Rep68-GFP
%-INPUT
no RBS 1 2 3 4 5 6 7 8 9
pRBS #


D
normalized %-INPUT (Rep68-GFP)
no RBS 1 2 3 4 5 6 7 8 9
pRBS #


E
normalized %-INPUT ratio (Rep68:Rep52)
no RBS 1 2 3 4 5 6 7 8 9
pRBS #


F
%-INPUT (rHSVEYFP-ICP4)
US1
ICP0 prom.
normalized %-INPUT (rHSVEYFP-ICP4)
US1
ICP0 prom.

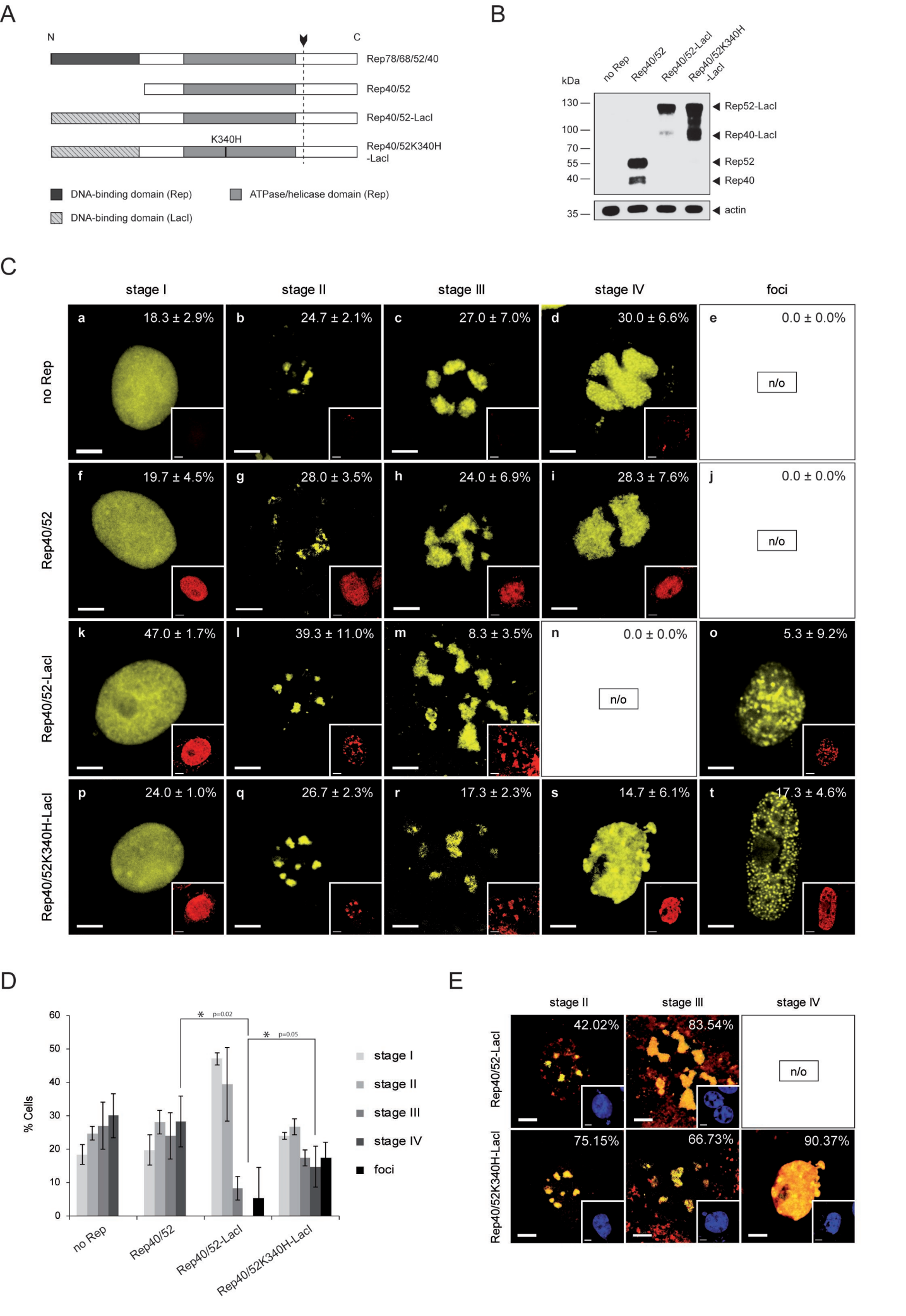

A
N
C
Rep78/68/52/40
Rep40/52
Rep40/52-LacI
K340H
Rep40/52K340H -LacI
DNA-binding domain (Rep)
ATPase/helicase domain (Rep)
DNA-binding domain (LacI)
B
kDa
no Rep
Rep40/52
Rep40/52-LacI
Rep40/52K340H -LacI
130
100
70
55
40
35
Rep52-LacI
Rep40-LacI
Rep52
Rep40
actin
C
stage I
stage II
stage III
stage IV
foci
no Rep
Rep40/52
Rep40/52-LacI
Rep40/52K340H-LacI
a 18.3 ± 2.9%
b 24.7 ± 2.1%
c 27.0 ± 7.0%
d 30.0 ± 6.6%
e 0.0 ± 0.0%
n/o
f 19.7 ± 4.5%
g 28.0 ± 3.5%
h 24.0 ± 6.9%
i 28.3 ± 7.6%
j 0.0 ± 0.0%
n/o
k 47.0 ± 1.7%
l 39.3 ± 11.0%
m 8.3 ± 3.5%
n 0.0 ± 0.0%
n/o
o 5.3 ± 9.2%
p 24.0 ± 1.0%
q 26.7 ± 2.3%
r 17.3 ± 2.3%
s 14.7 ± 6.1%
t 17.3 ± 4.6%
D
% Cells
60
50
40
30
20
10
0
p=0.02
p=0.05
stage I
stage II
stage III
stage IV
foci
no Rep
Rep40/52
Rep40/52-LacI
Rep40/52K340H-LacI
E
stage II
stage III
stage IV
Rep40/52-LacI
Rep40/52K340H-LacI
42.02%
83.54%
n/o
75.15%
66.73%
90.37%

Table 1. primer pairs used for ChIP-qPCR

#	forward	reverse
no RBS/US1	gcttccttgtttggagacca	gtccagtcaaactccccaaa
1	cacgtgcagcatctggtc	cgagcaacccacacacaat
2	atcgtgttgatctgctgcac	gaggagatcgccatcgtg
3	ccctcaagttcttcctcacg	ggagtcctggctgtctgttg
4	caaagcgcttcgaaactacc	gggtgtgatagacccacagg
5	gctaaatggcgactccttcc	cgatgtggtccggttgtag
6	gcctcgtggtggacgtatag	acatgatcctggctctcacc
7	aacggcctatccagcgtact	atatagcgcgtacgccaagg
8	gaccatcgcgttcattaaaaa	cgtgagggtgttgatgaagt
9	gcccgaccgaataaactgta	cctggatgtgcttcttaccg
ICP0 prom.	ataagttagccctggcccga	gctgcgtctcgctccg

Primers were generated using the online tool Primer3® (64, 65). The primer pair for the ICP0 promoter (ICP0 prom.) was described elsewhere (40). For all primer pairs the efficiency coefficients [E] were determined with serial dilutions of purified HSV-1 DNA (strain F) under the same qPCR conditions as described in Fig. 2C and used to standardize the %-INPUT calculations.